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Serological investigation of an outbreak of *Neospora caninum*-associated abortion in a dairy herd in southeastern United States

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Abstract

An outbreak of *Neospora caninum*-associated abortion occurred in a South Carolina dairy wherein greater than 10% of the herd aborted over a 4-month period. Of the total number of cows at mid-late gestation, nearly 40% (28/71) aborted while the remaining 60% (43/71) gave birth to normal calves. Immunohistochemical examination of brain tissue from a subset of aborted fetuses confirmed *N. caninum* as the causative agent of abortion in these animals. A variety of serological assays, including indirect fluorescence antibody test (IFAT), recombinant enzyme-linked immunosorbent assay (rELISA), ISCOM-ELISA, avidity ELISA, and *Neospora* agglutination test (NAT), were used to evaluate sera collected during the outbreak from 240 cows for antibodies to *N. caninum*. IFAT and ISCOM-ELISA testing showed that nearly 80% of the dairy cows had antibodies to *N. caninum*. NAT and rELISA had similar levels of seropositivity relative to IFAT and ISCOM-ELISA, but the percentage of positive sera was dependent on the cut-off value chosen. As indicated by κ coefficient statistical analysis, ISCOM-ELISA and IFAT exhibited the highest level of agreement in identifying *N. caninum*-positive and -negative cows. A decrease in the percentage of seropositive cows as determined by ISCOM-ELISA and IFAT with increasing cow age was noted. However, no

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significant difference was observed between cow age and abortion status. In addition to these tests, an avidity ELISA was performed on all sera with high ($\geq$0.4) ISCOM-ELISA readings. Avidity index (AI) increased with time post-abortion suggesting that most abortions were due to recent *N. caninum* infection. Of the cows at risk for abortion, the mean serological AI of aborting cows was significantly lower ($P < 0.05$) than mean serological AI of non-aborting cows. Published by Elsevier Science B.V.

Keywords: Neospora caninum; Abortion; Dairy cattle; Serologic assays; South Carolina

1. Introduction

The protozoan parasite *Neospora caninum* is well recognized as an important cause of abortion in dairy cows worldwide (for review see Dubey and Lindsay, 1996). The economic impact of this parasite on the dairy industry appears substantial (Dubey, 1999). Although abortions have been documented between 3 months — term, the highest incidence occurs at 5–6 months gestation (Anderson et al., 1991). At present, control of bovine neosporosis is difficult because many offspring of *N. caninum*-infected cows are born infected, but display no clinical signs of disease (Dubey and Lindsay, 1996). Whether the majority of infections occurs in utero or by ingestion of *N. caninum* oocysts is unknown. Although vertical transmission has been well documented (Björkman et al., 1996; Anderson et al., 1997), there is evidence that abortion storms may be a direct result of a point source oocyst infection (Thornton et al., 1994; McAllister et al., 1996).

Studies on the epidemiology of neosporosis have frequently involved testing of sera from dairy herds for antibodies (Ab) to *N. caninum*. A number of serological tests have been described, including indirect fluorescent antibody test (IFAT), *Neospora* agglutination test (NAT), and enzyme-linked immunosorbent assay (ELISA) using different *N. caninum* antigens or recombinant proteins (for review see Björkman and Uggla, 1999). These assays have been used to follow both natural and experimental *N. caninum* infections and to estimate the prevalence of neosporosis in dairy herds. Studies have shown that abortions are more prevalent in cows with high *N. caninum* Ab levels (Thurmond and Hietala, 1997; Wouda et al., 1998). The choice of cut-off value for each of these assays is also controversial (Björkman and Uggla, 1999). One additional shortcoming of most serological assays for *N. caninum* is that the presence of Ab only reflects exposure to the parasite and provides no information on the stage of infection (i.e. acute versus chronic). To avoid this drawback, an ELISA has been developed that measures the degree of functional affinity or avidity of IgG antibodies to *N. caninum* as a measure of recency of infection (Björkman et al., 1999). The purpose of the present study was to compare a variety of *N. caninum* serological assays, IFAT, ISCOM-ELISA, NAT, and recombinant ELISA (rELISA) using sera from a dairy herd that was in the midst of a *Neospora*-associated abortion storm. NAT and rELISA were also evaluated at several different cut-off values so that, similar to IFAT and ISCOM-ELISA, the appropriate cut-off indicative of a positive titer for these assays may be established. All sera with high ISCOM-ELISA values ($\geq$0.4) were further tested by avidity ELISA to gain insight on recency and possible source (oocyst versus congenital transmission) of infection.

2. Materials and methods

2.1. Description of dairy herd and abortion epidemic

A closed dairy herd located in northwestern South Carolina consisting of 240 Holstein cows age 2–10 years old experienced an abortion storm over a 4-month period between spring and summer 1999. During this time, 12% of the herd aborted ($n = 28$), while 18% produced normal births ($n = 43$). This was the first major outbreak of abortion in the herd. All cows had been vaccinated against IBR, BVD, and *Leptospira*. Abortions started in April, but the majority occurred in July and August and were mostly late term. A blood sample was collected on 27 July from all cows in the herd immediately following the first diagnosis of *N. caninum* abortion. The serum samples were shipped frozen to the USDA/ARS in Beltsville, MD, USA for testing.

2.2. Necropsy examination of aborted fetuses and serological testing of aborting cows

Six bovine aborted fetuses were submitted to the Clemson University Veterinary Diagnostic Center (CUVDC) from 12–14 July. The abortions had occurred during the seventh month of gestation. Ancillary diagnostic tests were performed to exclude other abortifacients including dark field examination of stomach contents for *Leptospira* organisms. Ocular fluids were tested for nitrates using Merck Nitrate test strips. Sections of lung, liver, kidney, heart, placenta, and brain were fixed in 10% neutral buffered formalin, subjected to a graded ethanol series, embedded in paraffin, sectioned at 5 μm, and were stained with hematoxylin and eosin (H&E) for light microscopy. Selected sections were processed for *N. caninum* immunohistochemical (IHC) examination (Lindsay and Dubey, 1989).

Serum samples from 11 aborting cows were tested at CUVDC for abortifacients. An IFAT was used at a 1:200 serum dilution for antibodies to *N. caninum* using whole tachyzoites (VMRD, Pullman, WA, USA). Assays for brucellosis, leptospirosis, infectious bovine rhinotracheitis (IBR), parainfluenza-3 (PI-3), and bovine viral diarrhea (BVD) were conducted using National Veterinary Services Laboratories (NVSL) protocols. The rapid agglutination plate (RAP) tests were run initially to screen for *Brucella abortus*. Five servers for *Leptospira* (*L. canicola, L. bratislava, L. grippo-typhosa, L. hardjo, L. icterohaemorrhagiae*) were measured by a microscopic agglutination test. Serum neutralization (SN) tests were performed beginning at 1:4 dilution to detect IBR, BVD, PI-3.

2.3. Indirect fluorescent antibody test (IFAT)

All 240 sera from adult cows bled on 27 July were tested by IFAT for *N. caninum* antibodies at a 1:25 dilution using described procedures (Dubey et al., 1988, 1996).

2.4. Neospora agglutination test (NAT)

The NAT was performed on all 240 sera using formalin-fixed *N. caninum* tachyzoites using described procedures (Romand et al., 1998). Sera were assayed by NAT starting at a 1:40 dilution; sera exhibiting a positive NAT reaction at this dilution were diluted further

and tested at 1:80, 1:160, and 1:320. The percentage of positive/negative sera was calculated at cut-off values of 40, 80, and 160.

2.5. ISCOM enzyme-linked immunosorbent assay (ISCOM-ELISA) and avidity ELISA

The ISCOM-ELISA was performed on all 240 sera as described (Björkman et al., 1997). A cut-off value of 0.20 was used to segregate positive and negative sera. Sera with an ISCOM-ELISA reading greater than 0.40 were tested by avidity ELISA using described procedures (Björkman et al., 1999). An avidity index (AI) was calculated for sera from 22 aborting cows and from 124 non-aborting cows. Avidity indices greater than 50 were considered indicative of chronic *N. caninum* infection, whereas AI less than 35 indicated a recent *N. caninum* infection (Björkman et al., 1999).

2.6. Recombinant ELISA (rELISA)

All 240 sera were assayed in rELISA using a combination of purified NCDG1 and NCDG2 (*N. caninum* dense granule) antigens as described (Jenkins et al., 1997). The *N. caninum* Ab titers were estimated using a standard curve generated from serial dilutions of a pool of positive control bovine sera as described (Jenkins et al., 1997). Percentage of positive/negative sera were calculated at three different cut-off values of 1000, 1500, and 2000.

2.7. Statistical analyses

Agreement between rELISA, NAT, ISCOM, and IFAT was estimated by κ coefficients (Cohen, 1960). Percent positive sera was also calculated for each assay and compared between different age groups of cows using Kruskal–Wallis one way analysis of variance on ranks and the Mann–Whitney rank sums test. Fisher's exact test were conducted for each assay to test for significant association between serological assay results and cows' abortion status. The likelihood of positive tests for aborting over non-aborting cows were calculated as odds ratios with 95% confidence intervals.

3. Results

Over a 4-month period, 39% (28/71) of cows at risk in a 240 cow South Carolina dairy herd aborted, while 61% (43/71) gave birth to normal calves. No significant relationship was observed between cow age and abortion status. Gross examination of six fetuses submitted to CUVDC revealed firm edematous lungs in all fetuses. Serosanguinous abdominal and thoracic fluid was noted in two of the fetuses. The hindlimbs of one fetus were hyperextended. No other gross lesions or developmental abnormalities were observed.

The microscopic lesions consisted primarily of encephalitis, myocarditis, and placentitis. Multifocal areas of necrosis with infiltrates of mixed inflammatory cells characterized the placentitis. The myocardium contained areas of necrosis accompanied by mixed inflammatory cell infiltrates. In the brain, multifocal areas of necrosis, gliosis, and rare perivascular collections of mononuclear cells were observed. In H&E sections, only one group of

Table 1
Comparison of serological assays for antibodies to *N. caninum* in a South Carolina dairy herd experiencing an *N. caninum*-associated abortion storm

Serological assay	Cut-off value	Positive/total	Percentage of positive sera
rELISA[a]	1000	213/240	89
	1500	177/240	74
	2000	152/240	63
NAT[b]	40	219/240	91
	80	148/240	62
	160	84/240	35
ISCOM-ELISA[c]		190/240	79
IFAT[d]		185/240	77

[a] Recombinant NCDG1 and NCDG2 enzyme-linked immunosorbent assay.
[b] *Neospora* agglutination test.
[c] ISCOM-enzyme-linked immunosorbent assay.
[d] Indirect fluorescent antibody test.

protozoa, adjacent to a glial nodule in the brain, was seen upon initial examination. By IHC examination, *N. caninum* tachyzoites were identified in cerebral lesions in three of six fetuses. *Leptospira* organisms were not detected in any of the fetuses. Ocular fluids tested negative for nitrate.

Serologic examination of aborting cows for brucellosis and PI-3 were negative. Median anti-IBR and anti-BVD titers were low (1:32) with maximum titers of 1:128 against both agents. Antibodies to *N. caninum* were detected at a 1:200 dilution of serum from all 11 cows tested by IFAT at CUVDC. Antibodies to *Leptospira* spp. were detected in some cows: *L. grippo-typhosa* — 6 negative, 5 positive with median titer of 1:200, maximum titer of 1:400; *L. bratislava* — 9 negative, 2 positive with median titer of 1:100, maximum titer of 1:100; *L. canicola* — 2 negative, 9 positive with median titer of 1:200, maximum titer of 1:400; *L. hardjo* — 1 negative, 10 positive with median titer of 1:200, maximum titer of 1:800; *L. icterohaemorrhagiae* — 8 negative, 3 positive with median titer of 1:200, maximum titer of 1:100.

Four different serological assays, including IFAT, ISCOM-ELISA, NAT, and rELISA were then used to evaluate sera collected from all 240 cows in the herd during the abortion outbreak. The IFAT results showed that 77% of the cows (185/240) had Ab to *N. caninum* (Table 1). A similar percentage of positive sera (79%) was shown by ISCOM-ELISA (Table 1). The percentage positive sera estimated by NAT and rELISA was dependent on the cut-off value chosen. Using a cut-off value of 1000, nearly 90% of cows were positive for *N. caninum* Ab in the rELISA; while only 63% were positive when a 2000 cut-off was used (Table 1). More than 90% of the sera were positive by NAT using a cut-off of 40. The percentage of positive sera decreased to 62% at a cut-off of 80 and decreased further to 35% at a cut-off of 160 (Table 1). IFAT and ISCOM-ELISA showed the best agreement (κ coefficient $= 0.72$) in identifying sera positive or negative for *N. caninum* Ab (Table 2). Agreement between each of the other tests was much lower (Table 2).

IFAT and ISCOM-ELISA testing of all cows in the herd showed that the percentage of seropositive cows decreased with age (data not shown). A comparison of aborting cows

Table 2
Agreement between recombinant enzyme-linked immunosorbent assay (rELISA), *Neospora* agglutination assay (NAT), ISCOM-ELISA, and indirect immuno-fluorescent antibody test (IFAT) analyses of sera from a dairy herd experiencing a *N. caninum*-associated abortion storm[a]

Assay 1	Assay 2	κ coefficient + A.S.E.[b]
rELISA (1000)	NAT (40)	0.15 ± 0.08
rELISA (1000)	NAT (80)	0.22 ± 0.05
rELISA (1000)	NAT (160)	0.08 ± 0.03
rELISA (1000)	ISCOM-ELISA	0.38 ± 0.08
rELISA (1000)	IFAT	0.45 ± 0.07
rELISA (1500)	NAT (40)	0.15 ± 0.06
rELISA (1500)	NAT (80)	0.29 ± 0.06
rELISA (1500)	NAT (160)	0.17 ± 0.05
rELISA (1500)	ISCOM-ELISA	0.23 ± 0.07
rELISA (1500)	IFAT	0.34 ± 0.07
rELISA (2000)	NAT (40)	0.13 ± 0.05
rELISA (2000)	NAT (80)	0.24 ± 0.06
rELISA (2000)	NAT (160)	0.18 ± 0.05
rELISA (2000)	ISCOM-ELISA	0.23 ± 0.06
rELISA (2000)	IFAT	0.34 ± 0.06
NAT (40)	ISCOM-ELISA	0.17 ± 0.07
NAT (40)	IFAT	0.16 ± 0.07
NAT (80)	ISCOM-ELISA	0.25 ± 0.06
NAT (80)	IFAT	0.31 ± 0.06
NAT (160)	ISCOM-ELISA	0.10 ± 0.03
NAT (160)	IFAT	0.11 ± 0.05
ISCOM-ELISA	IFAT	0.73 ± 0.05

[a] κ coefficients $\geq$0.4 are considered to indicate moderate agreement between tests; κ coefficients $\geq$0.8 are considered to indicate excellent agreement between tests. Values in parentheses are cut-off values analyzed for rELISA and NAT.
[b] Asymptomatic standard error.

($n = 27$) versus cows that delivered a normal calf ($n = 43$) showed that aborting cows had significantly higher ($P < 0.05$) ISCOM-ELISA readings than non-aborting cows (aborting $= 0.69 \pm 0.06$ versus non-aborting $= 0.47 \pm 0.06$). Although there was not a significant difference ($P > 0.05$) between the two groups, the percent seropositivity was higher in aborting cows than in non-aborting cows as measured by rELISA (all three cut-off values), IFAT, and ISCOM-ELISA (Table 3).

A positive relationship was observed between AI and the length of time from abortion to serum collection (Fig. 1). The mean AI of sera (79 ± 15) obtained from cows greater than 3 weeks post-abortion was significantly greater ($P < 0.0001$) than mean AI of sera (22 ± 7) collected from cows less than 3 weeks post-abortion. In the cows at risk (mid-late gestation) for which an AI was determined ($n = 46$), 70% of aborting cows had an AI less than 35 whereas only 27% of the non-aborting cows had an AI less than 35 (Table 4). The percentage of cows in the non-aborting group with AI greater than 50 was twice that in the aborting group (Table 4). In the at risk group, aborting cows had a significantly lower ($P < 0.05$) mean AI compared to non-aborting cows (39 ± 6 versus 60 ± 4). Mean AI of sera from the group of cows in the herd which were not at risk of abortion were also significantly higher ($P < 0.05$) than aborting cows (AI $= 55 \pm 3$).

Table 3
Comparison of rELISA, NAT, ISCOM-ELISA, and IFAT results between aborting ($n = 27$) and non-aborting ($n = 43$) cows at risk for abortion during the neosporosis-associated abortion storm

Serological assay	Cut-off value	Aborting cows Positive/total (% positive)	Non-aborting cows Positive/total (% positive)	Odds ratio[a]	P^*
rELISA	1000	25/27 (93)	36/43 (83)	2.4 (0.5, 12.7)	0.24
rELISA	1500	20/27 (74)	26/43 (60)	1.9 (0.7, 5.4)	0.18
rELISA	2000	19/27 (70)	22/43 (51)	2.3 (0.8, 6.3)	0.09
NAT	40	25/27 (93)	40/43 (93)	1.2 (0.4, 3.3)	0.71
NAT	80	18/27 (67)	27/43 (63)	0.9 (0.1, 6.0)	0.47
NAT	160	11/27 (41)	17/43 (40)	1.1 (0.4, 2.8)	0.56
ISCOM-ELISA		24/27 (89)	33/43 (77)	2.4 (0.6, 9.8)	0.17
IFAT		24/27 (89)	33/43 (77)	2.4 (0.6, 9.8)	0.17

[a] Values in parentheses are 95% confidence intervals.
* P value as calculated using Fisher's exact test of significance.

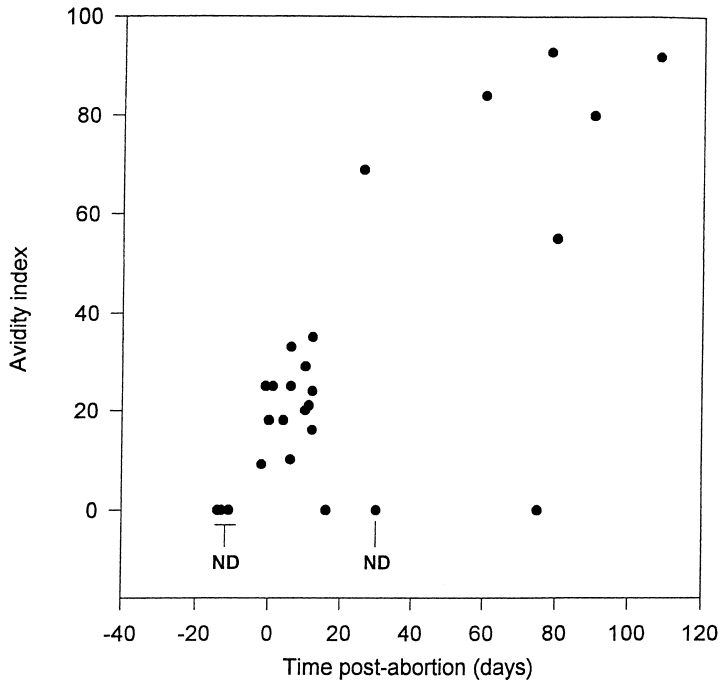

Fig. 1. Avidity index enzyme-linked immunosorbent assay testing of sera from aborting cows obtained at various times pre- or post-abortion. ND, avidity index not determined for four cows which exhibited low (<0.4) ISCOM-ELISA reading.

Table 4
Comparison of avidity ELISA values between aborting ($n = 20$) and non-aborting cows ($n = 26$) in South Carolina dairy herd experiencing a *N. caninum*-associated abortion storm[a]

Avidity index	Distribution by avidity index[b]	
	Aborting cows	Non-aborting cows
<35	14/20 (70)	7/26 (27)
35–50	0/20 (0)	2/26 (8)
>50	6/20 (30)	17/26 (65)

[a] AI determinations were attempted only for sera from at risk cows that produced an ISCOM-ELISA reading greater than 0.4 $O.D._{450}$ units. AI was calculated only for sera that produced an $O.D._{450}$ greater than 0.2 in the avidity ELISA (Bjorkman et al., 1999). AI < 35 is considered indicative of a recent *N. caninum* infection, whereas AI > 50 is considered indicative of chronic *N. caninum* infection.

[b] Numbers in parentheses are percentages of cows in either aborting or non-aborting groups that have serological AI values in the respective AI groups (<35, 35–50, >50).

4. Discussion

Serologic surveys indicate that the prevalence of *N. caninum* infection varies within and among herds, within a region, and among countries (reviewed by Dubey and Lindsay, 1996; Dubey, 1999). Some of these variations are related to the serologic test used and the cut-off values applied in each test. For example, the cut-off value employed in a serological assay is dependent on a range of factors such as antigen and conjugate used as well as recording system or apparatus employed. Thus, the cut-off can be influenced by varying the assay conditions which makes direct comparisons between results from different laboratories difficult (Björkman and Uggla, 1999). One purpose of the present study was to compare four different immuno-assays, rELISA, NAT, ISCOM-ELISA, and IFAT, to gain insight on the value of each test to characterize the serological response of a herd in the midst of a *N. caninum*-abortion storm.

In the present study, IFAT and ISCOM-ELISA testing showed that nearly 80% of the cows had Ab to *N. caninum*. Using the lowest cut-off value in NAT (40) and rELISA (1000) showed that over 90% of the cows had been exposed to *N. caninum*. As expected, increasing the cut-off value for NAT and rELISA resulted in a lower percentage of seropositive cows. The modest agreement of rELISA-1000 with both IFAT ($\kappa = 0.45$) and ISCOM-ELISA ($\kappa = 0.38$) indicates that a 1000 cut-off value for this test may be appropriate for discriminating positive from negative sera. However, the lack of good agreement between any of the tests, except between IFAT and ISCOM-ELISA, suggests the assays may be measuring Ab directed at different epitopes of *N. caninum*. For instance, the rELISA is directed at two dense granule proteins (NCDG1 and NCDG2), while the other assays detect Ab to surface and/or internal native *N. caninum* antigens. The lack of agreement among the serological assays is not surprising as the proportion of different *N. caninum* antigens may change during the course of infection.

Quite interesting were the AI readings of sera from aborting versus non-aborting animals. AI has been shown to reflect recency of *N. caninum* infection due to maturation of the immune response (Björkman et al., 1999). As a group, 70% of aborting cows in the present

study had AI less than 35 compared to less than half that percentage (27%) in non-aborting cows. The converse was observed with high AI values. Only 30% of aborting cows had AI greater than 50, while 65% of non-aborting cows had AI greater than 50. These data may indicate a greater risk of abortion with acute infections. It is clear, however, cows harboring a latent *N. caninum* infection can also abort due to reactivation of the parasite during gestation (Stenlund et al., 1999). Furthermore, the low AI values (AI $<$ 35) in a small number of non-aborting cows (7/26) indicate that not all acute infections lead to abortion.

The higher AI values in sera obtained from cows 3 weeks after abortion compared to lower AI values in sera from cows obtained about the time of abortion is consistent with previous studies on experimental *N. caninum* tachyzoite infections (Björkman et al., 1999). The present data suggest that most of the abortions in this storm were caused by a recent point source infection, possibly by *N. caninum* oocysts, rather than congenital infection. This conclusion is supported by a previous study that showed newborn calves infected in utero with *N. caninum* have high serological AI values (Björkman et al., 1999). High AI values were also associated with congenital infection in older cows (Björkman et al., 1999). The low AI values in sera from a small number ($n = 7$) of non-aborting cows indicate that these animals were also recently infected with *N. caninum*. These data imply that abortion is not always the outcome of *N. caninum* infection during mid-late gestation. The data also indicate that at least four of the abortions were not due to neosporosis because all of the assays conducted on sera from these cows were negative. It cannot be excluded that these animals were sampled too early during a possible *N. caninum* infection. The low serological titers against *Leptospira*, IBR, and BVD were probably due to vaccination and suggests, as for PI-3 and *Brucella abortus*, that these organisms were not responsible for the abortions.

Little is known of the life cycle and biology of *N. caninum* in cattle. Although vertical transmission or propagation is the major route of infection in cattle, post-natal transmission also occurs and is mandatory for introducing the parasite in closed herds. When and how the fetus becomes infected with *N. caninum* is unknown. In the only published study on oral inoculation with *N. caninum* oocysts, calves seroconverted within 4 weeks post-inoculation (DeMarez et al., 1999). However, the time period between *N. caninum* infection of the cow and its fetus is unknown, as is the time between fetal infection and abortion. Based on low AI values in a large number of aborting cows in the present study, it is possible that *N. caninum* oocyst infection during pregnancy leads to rapid transmission of the parasite to the fetus. Testing of this hypothesis awaits experimental infection of pre-parturient cows with *N. caninum* oocysts.

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